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[Document name] Detailed Description
[Title of invention] Fibroblast Growth Factor FGF-10

[Patent claims]

[Claim 1]

Recombinant DNA containing a nucleotide sequence

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encoding the polypeptide fibroblast growth factor whose amino acid sequence is represented by sequence number 1 or 2, or a nucleotide sequence complementary to the above nucleotide sequence.

[Claim 2]

The DNA described under Claim 1, containing the nucleotide sequence represented by sequence number 3 or 4 or a nucleotide sequence complementary to the above nucleotide sequence.

[Claim 3]

Expression vector containing the DNA described under Claim 1.

[Claim 4]

Transformant obtained by introducing the expression vector described under Claim 3 into a host.

[Claim 5]

The transformant described under Claim 4, in which the host is an animal cell or E. coli.

[Claim 6]

Method for producing recombinant fibroblast growth factor, characterized by the use of the transformant described under Claim 4.

[Claim 7]

Recombinant fibroblast growth factor that is a polypeptide containing the amino acid sequence represented by sequence number 1 or 2 or their major portions.

[Claim 8]

Recombinant fibroblast growth factor that is a polypeptide containing the amino acid sequence represented by sequence number 1 or 2 or their major portions, characterized by the fact that it is produced by the transformant described under Claim 5 and that has cell-growth-promoting activity.

[Claim 9]

Therapeutic agent for diseases of bone/cartilage or injuries of bone/cartilage, containing the recombinant fibroblast growth factor described under Claim 7 or 8 as active ingredient.

[Claim 10]

Wound-healing-promoting agent, containing the recombinant fibroblast growth factor described under Claim 7 or 8 as active ingredient.

[Detailed Description of the Invention]

[0001]

[Field in the Industry]

The present invention relates to a novel fibroblast growth factor (in the following, abbreviated as FGF) and a method for producing the same by recombination. Furthermore, it also relates to the medical applications of the factor.

[0002]

[Prior Art]

FGF was discovered in the 1970's as an angiogenic factor. Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) have been studied and their structures and wide-ranging cell-growth-promoting actions have been elucidated [D. Gospodarowics et al.: Nature Vol. 249, page 123 (1974); Burgess, W. H. and Maciag, T.: Annu. Rev. Biochem. Vol 58, pages 575-606 (1989); Suzuki, F.: Clinical Calcium, Vol. 4, pages 1516-1517 (1994)]. Currently, there are a total of 9 FGF species. They all have been cloned and their structures are known [Cell, Vol. 27, No. 9, pages 341-344 (1995)]. Existence of additional FGF species has been suggested.

[0003]

On the other hand, based on their wide-ranging cell-growth-promoting actions, aFGF and bFGF have been evaluated for their possible applications as promising therapeutic agents for the treatment of metabolic diseases of the nervous, cardiovascular and bone systems. However, the usefulness in the clinic so far has not been established. The same evaluation of novel FGF species is desired.

[Problems to be Solved by the Invention]

The objective of the present invention is to provide a method for industrial production of a recombinant protein of a novel FGF after identifying and then analyzing the gene.

[0004]

[Means of Solving the Problems]

The inventors actively investigated DNAs of unknown FGF species. As a result, they successfully obtained a DNA of a totally novel FGF (in the following, abbreviated as FGF-10), thereby achieving the present invention.

As described below, the present invention relates to the DNA encoding FGF-10, an expression vector containing the DNA, a transformant, a method for producing a recombinant protein by using the transformant, the recombinant protein, and medical applications of the recombinant FGF-10 protein.

[0005]

(1) A nucleotide sequence encoding a fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2, or a recombinant DNA containing a nucleotide sequence complementary to the above sequence.

(2) The DNA described under Claim 1, containing the nucleotide sequence represented by sequence number 3 or sequence number 4 or a nucleotide sequence complementary to the above sequence.

(3) An expression vector containing the DNA described under (1).

(4) A transformant obtained by introducing the expression vector described under (3) into a host cell.

(5) The transformant described under (4), in which the host cell is an animal or an E. coli cell.

(6) A method for producing a recombinant fibroblast growth factor, characterized by use of the transformant described under (4).

(7) A recombinant fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2 or its main portions.

(8) The recombinant fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2 or its main portions, characterized by the fact that it is produced by the transformant described under (5) and has cell-growth-promoting activity.

(9) A therapeutic agent for the treatment of diseases of bone/cartilage or injuries of bone/cartilage, containing the recombinant fibroblast growth factor described under (7) or (8) as active ingredient.

(10) A therapeutic agent for wound healing, containing the recombinant fibroblast growth factor described under (7) or (8) as active ingredient.

[0006]

In the present Detailed Description, definition of the technical terms is as follows.

FGF-10: A mammalian fibroblast growth factor containing the amino acid sequence represented by sequence number 1 or sequence number 2 or their major portions. The major portions include the amino acid sequence of mature protein after the signal (pre?) sequence or pro(?)sequence is deleted from the above sequence. In other words, it is the 179 amino acid residue sequence from glutamine (Gln)-37 to serine (Ser)-215 in sequence number 1 or

the 171 amino acid residue sequence from glutamine (Gln)-38 to serine (Ser)-208 in sequence number 2.

By currently known techniques proteins with some amino acids in the sequence represented by sequence number 1 or sequence number 2 or in the major portions deleted, substituted or added can be produced, and, based on knowledge obtained from random screening or studies on mutation of other members of the FGF family, mutant proteins with identical biological activity to that of FGF-10 of the present invention can be produced. As long as the mutant proteins have the activity of fibroblast growth factor, they should be covered by the present invention. FGF-10 is a protein with two sites for N-glycosylation: Asn-Ser-Ser (50-52) and Asn-Thr-Ser (203-205) in the amino acid sequence represented by sequence number 1 or Asn-Ser-Ser (51-53) and Asn-Thr-Ser (196-198) in the amino acid sequence represented by sequence number 2. In general, the biological activity does not depend on glycosylation. Glycosylation can be modified or removed by the selection of host cells. Proteins with modified glycosylation should be covered by the present invention as long as they have the fibroblast growth factor activity.

Fibroblast growth factor activity: At least one of the various biological activities of the FGF family including cell-growth-promoting activity such as cell growth-stimulating activity, hemopoietic progenitor growth-stimulating activity, angiogenic activity, etc., differentiation-modulating activity such as cell differentiation-inducing activity, extracellular matrix-modifying activity, etc., nerve-cell-survival-maintaining activity, etc. [Clinical Biochemistry, Vol. 38, No. 11, pages 219-221 (1994 Supplement Issue)]. The cell-growth-stimulating activity for epithelial cell-derived cell lines including the rat embryo-derived epithelial cell line (FRSK cells), seen with FGF-7, is also included in the activity.

Therapeutic agent for the treatment of injuries of bone/cartilage or diseases of bone/cartilage: A pharmaceutical preparation for the promotion of healing of physical injuries of bone/cartilage such as accident-caused fractures, surgical removal of bone/-cartilage, etc., or for the treatment of diseases with decreased osteogenesis as a major symptom. The following are its medical applications:

(1) bone deficiency-treating agent, (2) fracture-treating agent, (3) osteoporosis-treating agent, (4) chondral-tissue healing-promoting agent, (5) articular-chondral-tissue-healing- and treating agent, and (6) deforming-arthritis-treating agent.

Wound-healing-promoting agent: A pharmaceutical preparation for the promotion of healing of trauma, congelation, scalds, etc., caused by physical/chemical factors derived from accidents. Intractable dermatomuscular tissue disorders, such as radiation damage, skin ulcer derived from diabetes, bedsores, etc. are also included as targets of the treatment-promoting agent.

[0007]

In the following, the present invention is described in more detail.

[Production of FGF-10 gene]

The DNA encoding FGF-10 of the present invention can be obtained by known genetic engineering techniques. Specifically, mRNA can be isolated from animal tissues or cells, then double-stranded cDNA can be synthesized. The cDNA can be amplified by PCR using primers and the sequence can be determined. For all these experiments special kits are commercially available. Although there is no special limitation to tissues and culture cells as a source of mRNA, particularly rat embryo at about day 14 is preferably used. Since the expression level of the mRNA is relatively higher in lung and articular tissues, lung cells and culture cells derived from bone/cartilage also can be used. Commercially available poly(A) + RNA (from Clontech) from adult human lungs also can be easily and preferably used.

[0008]

It also can be cloned from cDNA or genomic DNA libraries from various species by using appropriate sequences from the DNA sequence encoding FGF-10 disclosed in the present Detailed Description as DNA probes.

DNA library is prepared as follows by standard procedures. 1. Lyophilized animal tissue is treated with RNase and protease, then high molecular weight DNA is obtained by precipitation. DNA extracts are commercially available (from Clontech, etc.). 2. By partial digestion with restriction enzyme (EcoRI, etc.), DNA fragments are obtained by ethanol precipitation. 3. The DNA fragments are inserted into λ phage by using DNA ligase.

4. By using a commercially available in-vitro packeting kit, packeting is performed thereby obtaining a DNA library.

DNA probes are selected based on highly distinct sequences from the DNA sequences encoding the FGF proteins disclosed in the present Detailed Description. They are chemically synthesized, then labeled with ^{32}P, etc.

[0009]

[Production of FGF-10 protein]

As the expression vector containing the FGF-10 cDNA thus obtained, plasmid or phage is selected, that can be amplified in appropriate host cells of E. coli, Bacillus subtilis, yeast, animal or insect. For example, the vector can be pBR322 or pBR325 derived from E. coli [Gene, Vol. 4, page 121 (1978)], pUB110 derived from Bacillus subtilis [Biochem. Biophys. Res. Commun. Vol. 112, page 678 (1983)], pCDM8 that is preferable for COS cells, etc. For the insertion of cDNA into plasmid, standard procedures are described in Molecular Cloning by T. Maniatis et al., Cold Spring Harbor Lab, page 239 (1982).

[0010]

The host cells are transformed by the introduction of the vector. There is no special limitation to the host cells, as long as they can produce FGF-10. Typical examples include bacteria such as E. coli, Bacillus subtilis (Bacillus bacteria), etc., yeast such as Saccharomyces, Torula, pikia(?), etc., animal cells such as COS cells, CHO cells, NSO cells, etc. Not only culture insect, fungus and plant cells, but also insects, mammals and plants containing the gene for the target protein are included in the hosts.

As a procaryotic cell production system, E. coli or Bacillus is generally used. In particular, Bacillus brevis with a reduced level of protease production is useful as an expression and secretion system [see Japanese Patent Application Publication Kokai No. Hei 6-296485, Japanese Patent Application Publication Kokai No. Hei 6-133782, Y. Sagiya et al.: Applied Microbiol. Biotechnol. Vol. 42, pages 358-363 (1994), etc.]

[0011]

Desired clones are selected from the transformants by known methods such as colony hybridization method [Gene, Vol. 10, page 63 (1980)] and DNA sequence determination method [Proc. Natl. Acad. Sci. USA, Vol. 74, page 560 (1977)]. Besides, clones also can

be selected by transient expression in COS cells followed by evaluation of the biological activity in the culture supernatants.

[0012]

The biological activity of the expressed FGF-10 can be easily detected by standard methods. For example, it can be evaluated by assaying for the growth-promoting activity for epithelial cells such as the known cell line FRSK.

[0013]

The plasmid containing the cloned DNA can be used directly, or after cut with restriction enzyme then inserted into an expression vector appropriate for selected host. FGF-10 protein can thus be produced in large quantities. There is no special limitation to expression method. All known techniques in this field can be used. For example, fusion expression, secretion expression or direct expression using bacteria, or expression using eukaryotic cells can be selected appropriately.

[0014]

The FGF-10 protein thus produced by recombinant technology can be purified by purification techniques generally used in the biochemical field. For example, appropriate combinations of ion exchange chromatography, gel filtration, reverse phase HPLC, ammonium sulfate precipitation, ultrafiltration, SDS-PAGE, etc. can be used. For FGFs, particularly affinity chromatography using heparin, etc. as a ligand, antibody column chromatography, etc. are preferably used for large scale purification. Antibodies against FGF-10 protein, both monoclonal and polyclonal, can be produced by known techniques. Specific antibodies against FGF-10 can be used not only for antibody column, but also for immunochemical quantitative assays such as ELISA, etc.

[0015]

[Mechanisms]

The FGF-10 protein produced by the above methods has various biological activities including cell-growth-promoting activity and thus can be used as a wound-healing-promoting agent, circulation deficiency-treating agent, nervous cell survival-maintaining agent, hair growth-promoting agent, etc. for medical applications. In particular, since its expression in chondral tissues of adult mammals, its applications as a bone disease-treating agent for the

treatment of fractures, etc. and as a therapeutic agent for the treatment of injuries of chondral and connective tissues are possible. Moreover, it also can be used as a reagent for research work on cell growth promotion.

[0016]

[Practical Examples]

In the following, the present invention is further described in detail by the way of practical examples. Nevertheless, the present invention is not to be limited to the examples.

[Practical Example 1]

Structural analysis of FGF-10 gene

Preparation of rat DNA library

From a whole, 14 day old Wistar rat embryo, mRNA was prepared by standard procedures [Chomoczynski et al.: Anal. Biochem. Vol. 162, pages 156-159 (1987)]. The rat embryo mRNA was used as template to prepare rat embryo cDNA using a random primer (6mer) as primer and Moloney murine leukemia virus reverse transcriptase. Specifically, rat embryo poly(A) + RNA (5 μg) was incubated in a solution containing 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals) and 0.5 μg of random primer (6mer) at 37°C for 60 minutes, thereby obtaining the cDNA.

[0017]

Preparation of primers common between FGF-3 and FGF-7

By comparing the amino acid sequences of known 7 human FGF species, 2 regions of amino acid sequences common between FGF-3 and FGF-7 (Tyr-Leu-Ala-Met-Asn-Lys and Tyr-Asn-Thr-Tyr-Ala-Ser) were selected, and 2 FGF primers as shown in Figure 1 were prepared.

[0018]

Amplification of rat FGF family DNAs

The rat embryo cDNA was used as template. The above 2 FGF primers and Taq DNA polymerase were used to amplify FGF family DNAs by the polymerase chain reaction (PCR) method. Specifically, a reaction solution (25 μL) containing an appropriate amount of cDNA, 0.05 unit/μL of Taq DNA polymerase (Wako Pure Chemicals) and 5 pmol/μL of the

above sense or antisense primer was subjected to 30 cycles of PCR. After the reaction, the solution was applied to 8% polyacrylamide gel electrophoresis, and the fraction with the desired size (about 110 bp) was eluted electrophoretically.

[0019]

Screening of rat FGF family DNAs

The FGF family DNAs thus amplified by using FGF primers were inserted into pGEM-T DNA vector (Promega). The resultant recombinant vector was transformed into E. coli (strain XL1-blue), thereby obtaining DNA clones. DNA Sequencer 373A (Applied Biosystems, Inc.) was used for the analysis of cDNA sequence.

By determining the nucleotide sequences of all the DNA clones, besides cDNAs for FGF-3 and FGF-7 that were known, a novel FGF cDNA encoding a peptide with similar amino acid sequence to known FGF-family peptides (about 50% similarity) was isolated. The clone was named FGF-10.

[0020]

Structural analysis of the entire coding region of rat FGF-10 cDNA

Primers were prepared based on the partial structure of FGF-10 cDNA identified in the above experiments. The entire coding region was obtained by using the Rapid Amplification of cDNA Ends (RACE) method [Frohman, PCR Protocols - A Guide to Methods and Applications, Academic Press, pp. 28-38 (1990)]. Details are described under (1)-(6).

[0021]

(1) Based on the partial structure of FGF-10 cDNA, primers A-D (Figure 2, sequence numbers 5, 6, 7 and 8) were prepared. In addition, primers X and Y were prepared by the RACE method (Figure 2, sequence numbers 9 and 10). (2) Random hexaoligonucleotide was used as primer to synthesize cDNA with reverse transcriptase using the rat embryo mRNA as template. A poly(A) sequence was added to the 3'-terminus with 3'-deoxynucleotidyl transferase in the presence of deoxyadenine triphosphate. The cDNA thus prepared was used as template to perform PCR using primers B and X. Furthermore, primers A and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide

sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named pFGF-10 (5'). (3) cDNA was synthesized with reverse transcriptase using the rat embryo mRNA as template and primer X. The cDNA thus produced was used as template to perform PCR using primers C and Y. Furthermore, primers D and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing the above partial sequences. It was named pFGF-10 (3'). (4) Based on the most upstream nucleotide sequence of pFGF-10 (5') and the most downstream nucleotide sequence of pFGF-10 (3'), primers E and F, respectively, were prepared (Figure 2, sequence numbers 11 and 12). (5) The rat embryo mRNA was used as template to synthesize single-stranded cDNA with reverse transcriptase using oligo(dT) as primer, which was then used as template to perform PCR using primers E and F. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined thereby obtaining clones with a nucleotide sequence containing the most upstream nucleotide sequence of pFGF-10 (5') and the most downstream nucleotide sequence of pFGF-10 (3'). Among them, 1 clone was selected, named pFGF-10. FGF-10 cDNA containing entire coding region, carried by the plasmid, was analyzed. (6) Taken together, the nucleotide sequence of sequence number 3 (804 bp) was determined.

[0022]

Determination of entire amino acid sequence of rat FGF-10

Based on the nucleotide sequence of FGF-10 cDNA obtained in the above experiments, it was known that the open reading frame of FGF-10 cDNA consists of 645 bp and that FGF-10 is a novel FGF consisting of 215 amino acids represented by sequence number 1. Analysis of the amino acid sequence revealed that it is a secreted protein with a signal sequence at the N-terminus. The mature protein is a polypeptide consisting of 179 amino acids between amino acid residue numbers 37-215. The Asn-Ser-Ser at 50-52 and Asn-Thr-Ser at 203-205 are N-glycosylation sites, suggesting that FGF-10 may be glycosylated.

[0023]

[Practical Example 2]

Expression of rat FGF-10 in mammalian cells

Construction of plasmids

The plasmid pFGF-10 (Figure 3) was digested with SphI and PstI, then a fragment containing the full length cDNA was separated by polyacrylamide gel electrophoresis. The fragment was ligated into pUC19 that had been digested with SphI and PstI, then transformed into E. coli strain JM109, thereby obtaining the plasmid pUC-F10 containing FGF-10 cDNA. pUC-F10 was digested with HindIII and XbaI to cut off a fragment containing FGF-10 cDNA, that was then ligated into the mammalian cell expression vector pCDM8 that had been digested with HindIII and XbaI, followed by transformation into E. coli strain MC1061/P3, thereby obtaining the plasmid pCDM8-F10SP containing FGF-10 cDNA downstream of CMV promoter.

On the other hand, since the nucleotide sequence upstream of the deduced translation start site in the FGF-10 cDNA was different from the Kozak consensus sequence, the translation efficiency of the mRNA was not considered to be high. Accordingly, to increase the translation efficiency, it was decided to perform mutation to convert the sequence upstream of the deduced translation start site to the Kozak consensus sequence [M. Kozak, The Journal of Cell Biology, Vol. 108, pages 229-241 (February, 1989)].

PCR was used to perform the mutation. pFGF-10 was used as template, and a sense primer having, as shown in Figure 4, the HindIII site at the 5'-end and Kozak consensus sequence, and an antisense, having the XbaI site at the 5'-end, were used (for reaction conditions, see Figure 4).

After the reaction, the PCR product was subjected to phenol-chloroform treatment, ether treatment then ethanol precipitation. After digestion with HindIII and XbaI, a fragment with about 700 bp was isolated by polyacrylamide gel electrophoresis. The fragment was ligated into the mammalian cell expression vector pCDM8 that had been digested with HindIII and XbaI. The vector was transformed into E. coli strain MC1061/P3. Among the resultant colonies, 4 clones were selected and the nucleotide sequences were analyzed by using DNA Sequencer (Perkin Elmer model 373).

The results showed that in all the clones the sequence upstream of the deduced translation start site was converted to the Kozak consensus sequence, and that there was no mutation on the amino acid sequence encoded. One clone was selected among these clones, named pCDM8-F10HX.

[0024]

[Practical Example 3]

Transformation of rat FGF-10-expressing plasmids in COS-1 cells

The rat FGF-10-expressing plasmids constructed in Practical Example 2, pCDM8-F10SP and pCDM8-F10HX, were prepared in large quantities by standard procedures, and purified by performing cesium chloride density gradient centrifugation twice. The two plasmids and pCDM8 as control were transformed into COS-1 cells by electroporation. The transformed cells were cultured for 24 hours in DMEM containing 10% bovine fetal serum that had been treated by lysine-Sepharose chromatography, then the medium was changed to serum-free DMEM. The culture was further performed for 96 hours. The culture supernatant thus obtained was centrifuged. The supernatant was stored at -80°C in aliquots.

[0025]

[Practical Example 4]

Confirmation of expression of FGF-10 mRNA in cartilage by in-situ hybridization

Preparation of probe: FGF-10 cDNA was inserted into the vector pGEM-T. The plasmid was transformed into E. coli strain JM-109. The bacteria were cultured in a large quantity. FGF-10 cDNA was highly purified by using Flexi Prep Kit from Pharmacia. The DNA sequence was confirmed by using Perkin Elmer 373A/DNA Sequencer. The cRNA probe was prepared by using DIG/RNA Labeling Kit (SP6/T7) from Boehringer.

[0026]

Preparation of tissue slice: A 3 week old female Wistar rat was sacrificed. A thigh bone and tibia with the joint in its original shape was collected. After the soft tissues were removed, it was trimmed into appropriate sizes then immediately soaked in fixing solution (4% paraformaldehyde). After fixing at 4°C overnight, dehydration was performed, then it was soaked in ash-removing solution (10% EDTA and 15% glycerol in PBS) for 4-5 days (the solution was replaced with fresh solution every day). The knee joint was trimmed into about 2 cm thick samples, which were then soaked in OCT compound and frozen with liquid

nitrogen. A cryostat was used to obtain 10 μm thick joint tissue slices, which were then mounted on a silane-coated glass slide.

Hybridization: After the above joint tissue slides were subjected to pretreatment (digestion with proteinase K, inactivation of endogeneous alkaline phosphatase with 0.2 M HCl, and acetylation with 0.1 M TEA and 0.25% acetic anhydride), they were dehydrated with ethanol. The above probe was diluted tenfold with hybridization solution (50% formamide, 10 mM Tris-HCl/pH 7.6, 200 μg/mL tRNA, 1 x Denhardt's solution, 10% Dextran sulfate, 600 mM NaCl, and 0.25% SDS), then 50 μL per slide was used. The sample was covered with a small sheet of parafilm. The hybridization was performed at 50°C for 16 hours. The excess probe was digested with RNase A. After washing with SSC, antibody binding and color development were performed.

[0027]

Antibody binding and color development: After the probe was washed off, the slide was soaked in blocking solution for 60 minutes. Alkaline-phosphatase-labeled anti-digoxigenin antibody (anti-digoxigenin-AP:Fab fragment, from Boehringer-Mannheim) was added onto the slide. After incubation at 37°C for 1 hour, the antibody solution was washed off. NBT, X-phosphate was added followed by incubation at 37°C for color development (12 hours). After the color was developed, the slide was soaked in color development stopping solution (10 mM Tris-HCl/pH 7.6, 1 mM EDTA/pH 8.0). After washing with distilled water, the slide was sealed with water.

Results: As shown in Figure 5 (A) and (B), color development was observed in chondral cells. Since FGF-10 mRNA is expressed in chondral cells, it is suggested that FGF-10 may be a factor involved in wound healing of bone and cartilage.

[0028]

[Practical Example 5]

Evaluation of cell-growth-promoting activity in FRSK cells

Cell culture: The rat epithelial cell line FRSK was cultured in culture flask with a culture area of 75 cm^2 in 15 mL of F-12 medium containing 10% of bovine fetal serum at 37°C in an atmosphere of 5% carbon dioxide/95% air. The cells were split at 1/10 once a week.

Expression of FGF-10 protein: FGF-10 was transiently expressed in COS-1 cells (see Practical Example 3). The culture supernatant was used in the following assays (in the following, the culture supernatants obtained from pCDM8-F10SP, pCDM8-F10HX and the control pCDM8 are represented by FGF-10/Sp, FGF-10/Hx and Bq, respectively).
DNA synthesis assay (tritium-labeled thymidine incorporation): The cells were cultured until subconfluence, then collected by trypsin treatment. A cell suspension at 10,000 cells/mL in the above medium was prepared, then distributed at 100 μL/well in a 96-well plate. The plate was incubated at 37°C in an atmosphere of 5% carbon dioxide/95% air. The medium was replaced with 100 μL of fresh medium once every 2 days. After 7 days' of culture, the medium was replaced with 100 μL of F-12 medium containing 0.1% of bovine fetal serum. After 24 hours, 25 μL of the COS supernatant was added. After 18 hours of culture at 37°C in an atmosphere of 5% carbon dioxide/95% air, 20 μL of F-12 medium containing 0.2 μCi of tritium-labeled thymidine was added, followed by incubation under the same conditions. After 4 hours, the medium was removed, and 50 μL of 2 N NaOH was added, followed by standing for 30 minutes to kill the cells. After neutralization with 1 N HCl, the cells were recovered with a cell harvester, and counted in Betaplate.

Results: As shown in Figure 6, compared to the control (Bq, 100%), the FGF-10 expression samples (Sp and Hx) both greatly enhanced the incorporation of tritium-labeled thymidine into FRSK cells (286% and 501%, respectively). Thus, it is shown that FGF-10 is a factor promoting the growth of epithelial cells.

[0029]

[Practical Example 6]

Structural analysis of human FGF-10 gene

Preparation of human DNA library

Commercially available human lung poly(A) + RNA (Clontech, Cat. No. 6524, from the whole lung of a male adult) was used as template to prepare human lung cDNA using a random primer (6mer) as primer and Moloney murine leukemia virus reverse transcriptase. Specifically, human lung poly(A) + RNA (5 μg) was incubated in a solution containing 300 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals) and 0.5 μg of random primer (6mer) at 37°C for 60 minutes, thereby obtaining the cDNA.

[0030]

Preparation of primers for amplifying human FGF-10 DNA and amplification of human FGF family DNAs

The 2 FGF primers shown in Figure 1 and used in Practical Example 1 (Tyr-Leu-Ala-Met-Asn-Lys and Tyr-Asn-Thr-Tyr-Ala-Ser) were selected and used for the amplification of human FGF-10 DNA.

Human lung cDNA was used as template. The above 2 FGF primers and Taq DNA polymerase were used to amplify FGF family DNAs by the polymerase chain reaction (PCR) method. Specifically, a reaction solution (25 μL) containing an appropriate amount of cDNA, 0.05 unit/μL of Taq DNA polymerase (Wako Pure Chemicals) and 5 pmol/μL of the above sense or antisense primer was subjected to 30 cycles of PCR. After the reaction, the solution was applied to 8% polyacrylamide gel electrophoresis, and the fraction with the desired size (about 110 bp) was eluted electrophoretically.

[0031]

Screening of human FGF family DNAs

The FGF family DNAs thus amplified by using FGF primers were inserted into the pGEM-T DNA vector (Promega). The resultant recombinant vector was transformed into E. coli (strain XL1-blue), thereby obtaining DNA clones. DNA Sequencer 373A (Applied Biosystems, Inc.) was used for the analysis of the cDNA sequence.

By determining the nucleotide sequences of all the DNA clones, a cDNA encoding a peptide identical to that of rat FGF-10 was identified. This DNA is considered to encode the human FGF-10.

[0032]

Structural analysis of entire coding region of human FGF-10 cDNA

A cDNA containing the entire coding region of human FGF-10 was amplified and analyzed as in Practical Example 1, thereby obtaining the nucleotide sequence represented by sequence number 4. Details are described under the following sections (1)-(6).

[0033]

(1) Based on the partial structure of human FGF-10 cDNA, primers A' and D' (Figure 2, sequence numbers 13 and 14) were prepared. Primers B, C, X and Y were identical to those in Practical Example 1 (Figure 2, sequence numbers 5, 6, 7 and 8).
(2) Random hexaoligonucleotide was used as primer to synthesize cDNA with reverse transcriptase using the human lung mRNA as template. A poly(A) sequence was added to the 3'-terminus with 3'-deoxynucleotidyl transferase in the presence of deoxyadenine triphosphate. The cDNA thus prepared was used as template to perform PCR using primers A' and X. Furthermore, primers B and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining a clone containing portions of the above partial sequences. The clone was named phFGF-10 (5'). (3) cDNA was synthesized with reverse transcriptase using the human lung mRNA as template and primer X. The cDNA thus produced was used as template to perform PCR using primers C and X. Furthermore, primers D' and Y were used to perform PCR. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined to obtain a clone containing portions of the above partial sequence. It was named phFGF-10 (3'). (4) Since the nucleotide sequence of phFGF-10 (5') upstream of the translation region was also present in the rat gene, primer E (Figure 2, sequence number 12) was used as primer for the 5'-end. On the other hand, based on the most downstream nucleotide sequence of phFGF-10 (3'), primer F' was prepared as primer for the 3'-end (Figure 2, sequence number 15). (5) The human lung mRNA was used as template to synthesize single-stranded cDNA with reverse transcriptase using oligo(dT) as primer, which was then used as template to perform PCR using primers E and F'. The resultant amplified fragments were inserted into pGEM-T, then transformed into E. coli (strain XL1-blue) thereby obtaining clones. The nucleotide sequences of several clones were determined thereby obtaining clones with a nucleotide sequence containing the most upstream nucleotide sequence of phFGF-10 (5') and the most downstream nucleotide sequence of phFGF-10 (3'). Among them, one clone was selected, named phFGF-10. Human FGF-10 cDNA containing the entire coding region, carried by the plasmid, was analyzed. (6) Taken together, the nucleotide sequence of sequence number 4 (690 bp) was determined.

[0034]

Determination of entire amino acid sequence of human FGF-10

Based on the nucleotide sequence of the cDNA obtained in the above experiments, it was known that the open reading frame of human FGF-10 cDNA consists of 624 bp and that human FGF-10 is a polypeptide consisting of 208 amino acids represented by sequence number 2. Analysis of the amino acid sequence revealed that it is a secreted protein with a signal sequence at the N-terminus. The mature protein is a polypeptide consisting of 171 amino acids between amino acid residue numbers 38-208. The Asn-Ser-Ser at 51-53 and Asn-Thr-Ser at 196-198 are N-glycosylation sites, suggesting that the protein may be glycosylated.

[0035]

[Practical Example 7]

Expression and purification of mature protein of human FGF-10

pFGF-10 was used as template to perform 15 cycles of PCR using the following primer pair (sequence numbers 16 and 17), then phenol-chloroform treatment and ethanol precipitation were performed. After digestion with NdeI and BamHI and subsequent polyacrylamide gel electrophoresis, a band with the targeted size was isolated, thereby obtaining a DNA fragment (a) corresponding to the amino acid sequence of the mature protein of human FGF-10 cDNA. On the other hand, the E. coli expression vector pET11c (Stratagen) was digested with NdeI and BamHI, then the vector DNA (b) was obtained by agarose gel electrophoresis. (a) and (b) were ligated with each other, then transformed into E. coli strain JM109, thereby obtaining clones. Among the clones, a plasmid with (a) in the right orientation was isolated and the DNA sequence was determined, which was named pET-hFGF-10. The plasmid was transformed into E. coli BL21 (DE3). One of the resultant recombinant clones was named BL21 (DE3)/pET-hFGF-10, which was used for the production of human FGF-10.

[0036]

Four flasks of the bacteria containing BL21 (DE3)/pET-hFGF-10 were cultured in 10 mL each of LB medium containing 100 μg/mL of ampicillin at 37°C overnight. The next day, the entire content of each flask was added to 500 mL of TB medium containing 100 μg/mL of (ampicillin), and the mixture was cultured at 37°C with agitation. After the OD600 reached 0.8, IPTG was added to a final concentration of 1 mM. The culture temperature was decreased to 28°C and the culture was continued for 6 hours.

[0037]

The culture medium was separated by centrifugation. The resultant bacteria were washed once with 50 mM Tris-HCl, pH 8.0, then suspended in 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 2 μg/mL leupeptin, 2 μg/mL pepstatin and 1 mM PMSF. The bacteria were broken by sonication, then centrifuged in a Beckman J2-21M/E high-speed refrigerated centrifuge with a JA-20 rotor at 15,000 rpm for 1 hour. The supernatant was collected. HiTrap Heparin (5 mL, from Pharmacia) was equilibrated with 50 mM Tris-HCl, pH 8.0, then the above supernatant was applied. The resin was washed with 50 mM Tris-HCl, pH 8.0 until the A260 of the eluate returned to the base line. A continuous NaCl concentration gradient up to 3 M was applied to elute the proteins. A protein with a molecular weight of about 19 kDa, that seemed to be the recombinant human FGF-10, was eluted at about 1.2 M NaCl. The flow rate was 2 mL/minute.

[0038]

Subsequently, the above eluate was diluted twofold with 50 mM Tris-HCl, pH 8.0, then applied to Hi Trap SP (5 mL, from Pharmacia). After washing with 50 mM Tris-HCl, pH 8.0, a continuous NaCl concentration gradient up to 2 M was applied to elute proteins. A protein with a molecular weight of about 19 kDa, that looked to be the recombinant human FGF-10, was eluted at about 1.2 M NaCl. The flow rate was 2 mL/minute. The above eluate was dialyzed against PBS (-), then Pyrosep 1C (from Daicel Kogyo) was added in an amount of 1/10 in order to remove the endotoxin. After agitation at 4°C for 2 hours, the supernatant was recovered. The endotoxin was assayed by using Endospecy(?) ES-6 (Seikagaku Kogyo), and the level was found to be below the detection limit. Protein was assayed by using the Protein Assay Kit (Bio Rad), and a total of 3.5 mg of protein was obtained.

[0039]

[Practical Example 8]

Effects of FGF-10 on bone tissues

In the following, the pharmaceutical preparation of the bone(cartilage)-disease-treating agent and the effects on bone (cartilage) tissue genesis and repair are described by the way of pharmaceutical preparation and testing examples.

Pharmaceutical Preparation: The purified mature protein of FGF-10 produced in Practical Example 7 was prepared as an aqueous solution for injection. Specifically, human FGF-10 (2.12 mg) was dissolved in saline (1 mL), then used for the following experiments.

[0040]

In vivo evaluation of efficacy: effects of FGF-10 on bone tissue

The aqueous solution of FGF-10 prepared in Pharmaceutical Preparation Example 1 was injected intramedullarly in the tibia using a microsyringe with 27 G needle in 3 groups of 4 week old male Wistar rats (body weight: 94-120 g), each 3-4 animals, after anesthetizing them with ether, at doses of 10.6, 21.2 and 0 (as control) μg.

[0041]

After 4 days, the animals were sacrificed, and the tibias were removed. Soft x-ray photographs of the tibias were taken, and the photos were input into personal computer using scanner. An image processing program was used to observe images of osteogenesis.

[0042]

The results of FGF-10 and control groups are shown in Figure 7 and Figure 8, respectively. In the human FGF-10 administration groups, increase in osteogenesis was recognized. The results of intramedullary osteogenesis on day 4 after administration of human FGF-10, evaluated by image analysis of soft x-ray photos, are shown in Table 1.

[Table 1]

FGF-10 (μg) number of animals	0 3	10.6 4	21.2 4
number of animals which showed osteogenesis in comparison to the control animals	--	4	4

These results show that human FGF-10 exhibits genesis- and repair-promoting activity for bone/cartilage tissues, important for the treatment of bone/cartilage diseases.

[0043]

[Effects of the Invention]

As clearly shown by the above results, human FGF-10 was found to have excellent action of generation and regeneration of bone/cartilage tissues and to be useful for the treatment of diseases of bone/cartilage tissues. Thus, the present therapeutic agent for diseases of bone/cartilage is useful for the treatment of various diseases of bone/cartilage tissues, for example, (1) the repair of damaged cartilage caused by deforming arthritis or arthritis as a result of autoimmune diseases such as chronic rheumatoid arthritis, etc., (2) repair of damaged cartilage caused by traumas or osteochondritis dissecans, (3) treatment of spinal diseases such as degenerated or herniated disk, (4) promotion of chondrogenesis after osteotomy, (5) repair after fractures, (6) repair after bone damage, (7) promotion of osteogenesis at sites where local bone reduction due to osteoporosis, etc. has been recognized.

Thus, the present invention provides pharmaceutical preparations using the novel factor, as well as the DNA encoding FGF-10, the expression vectors carrying the DNA, the transformants, the method for producing the recombinant proteins using the above reagents, and the recombinant proteins.

[0044]

[Sequence Table]

sequence number: 1

sequence length: 215

sequence form: amino acid

topology: linear chain

sequence type: peptide

origin

species name: rat

sequence:

```
Met Trp Lys Trp Ile Leu Thr His Cys Ala Ser Ala Phe Pro His Leu
  1               5                  10                  15
Pro Gly Cys Cys Cys Cys Phe Leu Leu Leu Phe Leu Val Ser Ser Val
                 20                  25                  30
Pro Val Thr Cys Gln Ala Leu Gly Gln Asp Met Val Ser Pro Glu Ala
             35                  40                  45
Thr Asn Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Phe
     50                  55                  60
Ser Ser Pro Ser Ser Ala Gly Arg His Val Arg Ser Tyr Asn His Leu
 65                  70                  75                  80
Gln Gly Asp Val Arg Trp Arg Lys Leu Phe Ser Phe Thr Lys Tyr Phe
                 85                  90                  95
Leu Lys Ile Glu Lys Asn Gly Lys Val Ser Gly Thr Lys Lys Glu Asn
            100                 105                 110
Cys Pro Tyr Ser Ile Leu Glu Ile Thr Ser Val Glu Ile Gly Val Val
        115                 120                 125
Ala Val Lys Ala Ile Asn Ser Asn Tyr Tyr Leu Ala Met Asn Lys Lys
    130                 135                 140
Gly Lys Leu Tyr Gly Ser Lys Glu Phe Asn Asn Asp Cys Lys Leu Lys
145                 150                 155                 160
Glu Arg Ile Glu Glu Asn Gly Tyr Asn Thr Tyr Ala Ser Phe Asn Trp
                165                 170                 175
Gln His Asn Gly Arg Gln Met Tyr Val Ala Leu Asn Gly Lys Gly Ala
            180                 185                 190
Pro Arg Arg Gly Gln Lys Thr Arg Arg Lys Asn Thr Ser Ala His Phe
        195                 200                 205
Leu Pro Met Val Val His Ser
    210                 215
```

[0045]

sequence number: 2

sequence length: 208

sequence form: amino acid

topology: linear chain

sequence type: peptide

origin

species name: human

sequence:

```
Met Trp Lys Trp Ile Leu Thr His Cys Ala Ser Ala Phe Pro His Leu
 1               5                  10                  15
Pro Gly Cys Cys Cys Cys Cys Phe Leu Leu Leu Phe Leu Val Ser Ser
            20                  25                  30
Val Pro Val Thr Cys Gln Ala Leu Gly Gln Asp Met Val Ser Pro Glu
        35                  40                  45
Ala Thr Asn Ser Ser Ser Ser Ser Phe Ser Ser Pro Ser Ser Ala Gly
    50                  55                  60
Arg His Val Arg Ser Tyr Asn His Leu Gln Gly Asp Val Arg Trp Arg
65                  70                  75                  80
Lys Leu Phe Ser Phe Thr Lys Tyr Phe Leu Lys Ile Glu Lys Asn Gly
                85                  90                  95
Lys Val Ser Gly Thr Lys Lys Glu Asn Cys Pro Tyr Ser Ile Leu Glu
            100                 105                 110
Ile Thr Ser Val Glu Ile Gly Val Val Ala Val Lys Ala Ile Asn Ser
        115                 120                 125
Asn Tyr Tyr Leu Ala Met Asn Lys Lys Gly Lys Leu Tyr Gly Ser Lys
    130                 135                 140
Glu Phe Asn Asn Asp Cys Lys Leu Lys Glu Arg Ile Glu Glu Asn Gly
145                 150                 155                 160
Tyr Asn Thr Tyr Ala Ser Phe Asn Trp Gln His Asn Gly Arg Gln Met
                165                 170                 175
Tyr Val Ala Leu Asn Gly Lys Gly Ala Pro Arg Arg Gly Gln Lys Thr
            180                 185                 190
Arg Arg Lys Asn Thr Ser Ala His Phe Leu Pro Met Val Val His Ser
        195                 200                 205
```

[0046]

sequence number: 3

sequence length: 804 bp

sequence form: nucleic acid

number of chains: double-stranded chain

topology: linear chain

sequence type: cDNA

origin

species name: rat

existing position: 109-753

method for determining the characteristics: E

sequence:

TAACCAGTAG CCATCACCTC CAGCTGTCTC TTTGCCTCGC ACCAGGTCTT ACCCTTCCAG 60
TATGTTCCTT CTGATGAGAC AATTTCCAGT GCC■

TGG ATA CTG ACA CAT TGT GCC TCA GCC TTT CCC CAC CTG CCG GGC TGC 165
TGT TGC TGC TTC TTG TTG CTC TTC TTG GTG TCT TCC GTC CCT GTC ACC 213
TGC CAA GCT CTT GGT CAG GAC ATG GTG TCA CCG GAG GCT ACC AAC TCC 261
TCT TCC TCC TCC TCT TCC TCC TCC TCG TCC TCT TCC TTC TCC TCT CCT 309
TCC AGC GCG GGG AGG CAT GTG CGG AGC TAC AAT CAC CTC CAG GGA GAT 357
GTC CGC TGG AGA AAG CTG TTC TCC TTC ACC AAG TAC TTT CTC AAG ATT 405
GAA AAG AAC GGC AAG GTC AGC GGG ACC AAG AAG GAA AAC TGT CCG TAC 453
AGT ATC CTA GAG ATA ACA TCA GTG GAA ATC GGA GTT GTT GCC GTC AAA 501
GCC ATT AAC AGC AAC TAT TAC TTA GCC ATG AAC AAG AAG GGG AAA CTC 549
TAT GGC TCA AAA GAA TTT AAC AAT GAC TGT AAA CTG AAA GAG AGG ATA 597
GAG GAA AAT GGA TAC AAC ACC TAT GCA TCT TTT AAC TGG CAG CAC AAC 645
GGC AGG CAA ATG TAT GTG GCA TTG AAT GGA AAA GGA GCT CCC AGG AGA 693
GGA CAA AAA ACA AGA AGG AAA AAC ACC TCC GCT CAC TTC CTC CCC ATG 741
GTG GTC CAC TCA TAGAAGA AGGCACCGTT GGTGGATGCA GTACAACCAA TGACTCTTTG 800
CCAA

[0047]

sequence number: 4
sequence length: 690 bp
sequence form: nucleic acid
number of chains: double-stranded
topology: linear chain
sequence type: cDNA
origin
species name: human
sequence:

TGG AAA TGG ATA CTG ACA CAT TGT GCC TCA GCC TTT CCC CAC CTG CCC 106
GGC TGC TGC TGC TGC TGC TTT TTG TTG CTG TTC TTG GTG TCT TCC GTC 154
CCT GTC ACC TGC CAA GCC CTT GGT CAG GAC ATG GTG TCA CCA GAG GCC 202
ACC AAC TCT TCT TCC TCC TCC TTC TCC TCT CCT TCC AGC GCG GGA AGG 250
CAT GTG CGG AGC TAC AAT CAC CTT CAA GGA GAT GTC CGC TGG AGA AAG 298
CTA TTC TCT TTC ACC AAG TAC TTT CTC AAG ATT GAG AAG AAC GGG AAG 346
GTC AGC GGG ACC AAG AAG GAG AAC TGC CCG TAC AGC ATC CTG GAG ATA 394
ACA TCA GTA GAA ATC GGA GTT GTT GCC GTC AAA GCC ATT AAC AGC AAC 442
TAT TAC TTA GCC ATG AAC AAG AAG GGG AAA CTC TAT GGC TCA AAA GAA 490
TTT AAC AAT GAC TGT AAG CTG AAG GAG AGG ATA GAG GAA AAT GGA TAC 538
AAT ACC TAT GCA TCA TTT AAC TGG CAG CAT AAT GGG AGG CAA ATG TAT 586
GTG GCA TTG AAT GGA AAA GGA GCT CCA AGG AGA GGA CAG AAA ACA CGA 634
AGG AAA AAC ACC TCT GCT CAC TTT CTT CCA ATG GTG GTA CAC TCA TAGAG 684
GAAGGC 690

[0048]

sequence number: 5

sequence length: 22 bp

sequence form: nucleic acid

number of chains: single-stranded chain

topology: linear chain

sequence: GATGCATAGG TATTGTATCC AT

sequence number: 6

sequence length: 21 bp

sequence form: nucleic acid

number of chains: single-stranded chain

topology: linear chain

sequence: TCCATTTTCC TCTATCCTCT C

sequence number: 7

sequence length: 20 bp

sequence form: nucleic acid

number of chains: single-stranded chain

topology: linear chain

sequence: AGAAGGGGAA ACTCTATGGC

[0049]

sequence number: 8

sequence length: 21 bp

sequence form: nucleic acid

number of chains: single-stranded chain

topology: linear chain

sequence: GACTGTAAAC TGAAAGAGAG G

sequence number: 9
sequence length: 32 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GCGAGCTCAA GCTTTTTTTT TTTTTTTTTT TT

sequence number: 10
sequence length: 18 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GCGAGCTCAA GCTTTTTT

[0050]
sequence number: 11
sequence length: 20 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: CTTCCAGTAT CATCCTTCTG

sequence number: 12
sequence length: 20 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GGCAAAGAGT CATTGGTTGT

sequence number: 13
sequence length: 22 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GATGCATAGG TATTGTATCC AT

[0051]
sequence number: 14
sequence length: 22 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GAAACTCTAT GGCTCAAAAG AA

sequence number: 15
sequence length: 20 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GTACACTCAT AGAGGAAGGC

sequence number: 16
sequence length: 34 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: GGGAATTCCA TATGCTTGGT CAGGACATGG TGTC

sequence number: 17
sequence length: 29 bp
sequence form: nucleic acid
number of chains: single-stranded chain
topology: linear chain
sequence: CGCGGATCCG CTATGCATGC AACGCGTTG

[0052]

[Brief Legends to the Figures]

[Figure 1]

It shows two primers used for the cloning of FGF-10, which are common to FGF-3, FGF-7 and FGF-10: (A) Tyr-Leu-Ala-Met-Asn-Lys; (B) Tyr-Asn-Thr-Tyr-Ala-Ser.

[Figure 2]

It shows primers used for the isolation of FGF-10 cDNA by the method of Rapid Amplification of cDNA Ends (RACE).

[Figure 3]

It shows a summary of the construction of plasmids from the plasmid pFGF-10 to the plasmid pCDM8-F10SP and finally to the plasmid pCDM8-F10HX.

[Figure 4]

It shows primers and PCR conditions used to convert the sequence upstream of the translation start site to the Kozak consensus sequence.

[Figure 5]

It shows the expression of FGF-10 mRNA in articular tissue detected by in situ hybridization: (A) micrograph of an articular cartilage specimen; (B) micrograph of an apophysiary cartilage specimen.

[Figure 6]

It shows a graph of the incorporation of tritium-labeled thymidine into FRSK cells. Bq, Sp and Hx of the horizontal axis represent control and supernatant samples of FGF-10-expressing COS cell culture, respectively, while the vertical axis represents the cell-associated radioactivity.

[Figure 7]

It shows a processed image of X-ray photography of tibial cartilage from a FGF-10-treated animal in a test example.

[Figure 8]

It shows a processed image of x-ray photography of tibial cartilage from a control animal in the test example.

[Document name] Figures

[Figure 1]

sense primer

```
        Tyr Leu Ala Met Asn Lys
    5'- TAC CTA GCA ATG AAC AA -3'
          T   C   C       T
              G   G
              T   T
```

antisense primer

```
        Tyr Asn Thr Tyr Ala Ser
    3'- ATA TTA TGA ATA CGA AG  -5'
          G   G   C   G   C
                  G       G
                  T       T
```

[Figure 2]

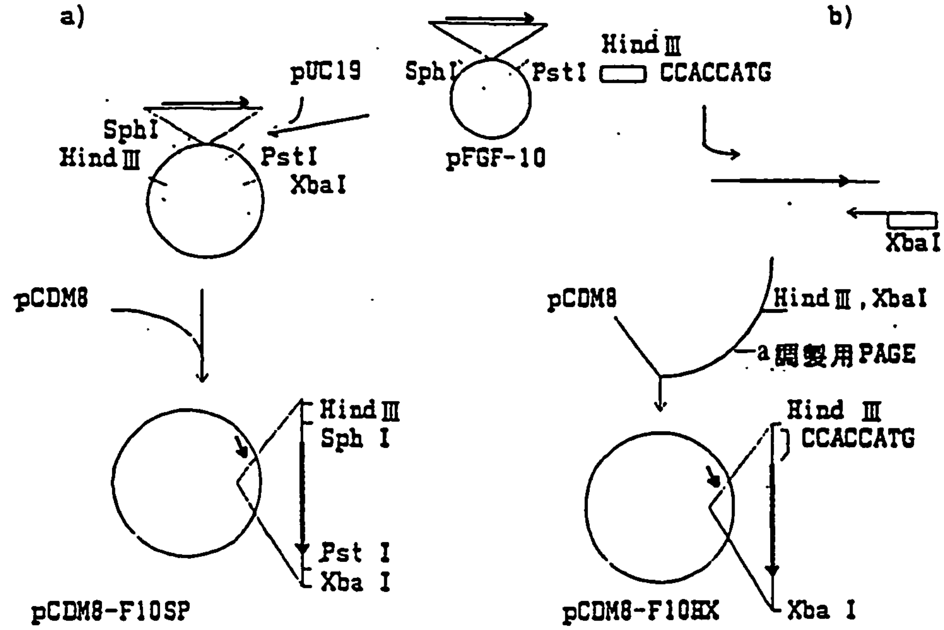
5' RACE method
A: 5'-CCT CTC TTT CAG TTT ACA GTC -3'
A':5'-GAT GCA TAG GTA TTG TAT CCA T-3'
B: 5'-TCC ATT TTC CTC TAT CCT CTC -3'
X: 5'-GCG AGC TCA AGC TTT TTT TTT TTT TTT TTT TT-3'
Y: 5'-GCG AGC TCA AGC TTT TTT -3'

3' RACE method
C: 5'-AGA AGG GGA AAC TCT ATG GC -3'
D: 5'-GAC TGT AAA CTG AAA GAG AGG -3'
D':5'-GAA ACT CTA TGG CTC AAA AGA A-3'
X: 5'-GCG AGC TCA AGC TTT TTT TTT TTT TTT TTT TT-3'
Y: 5'-GCG AGC TCA AGC TTT TTT -3'

for amplifying total sequence
E: 5'-CTT CCA GTA TGT TCC TTC TG-3'
F: 5'-GGC AAA GAG TCA TTG GTT GT-3'
F':5'-GTA CAC TCA TAG AGG AAG GC-3'

[Figure 3]

a: Preparative PAGE



[Figure 4]

Nucleotide sequences of the primers used for converting the region upstream of the deduced translation start site to Kozak consensus sequence

name	number of nucleo-tides	sequence (5'→3')
F10HS	35mer	TTTTAAGCTT CCACC ATGTGGAAGTGGATACTGAC
F10XR	27mer	AAAATCTAGA GTCATTGGTTGTACTGC

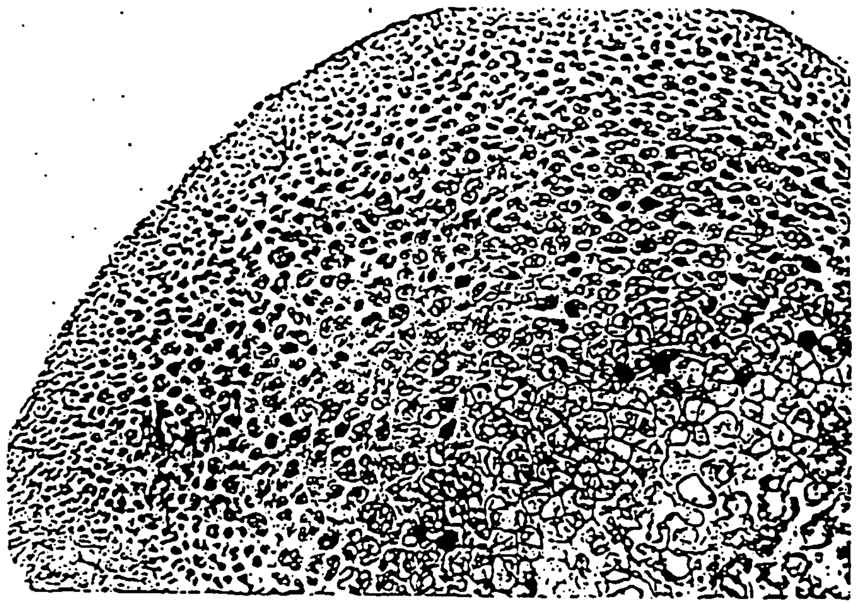
reaction conditions:

pFGF-10(0.5 μg/μl)	2	μl
10xPCR buffer	10	
10 μM F10HS	2.5	
10 μM F10XR	2.5	
dNTP mix(TaKaRa)	8	
dH2O	74.5	
AmpliTaq	0.5	/100 μl

94°C	30 sec	
94°C	60 sec	10 times
56°C	60 sec	
72°C	60 sec	
72°C	9 minutes	

mutation-introducing fragments

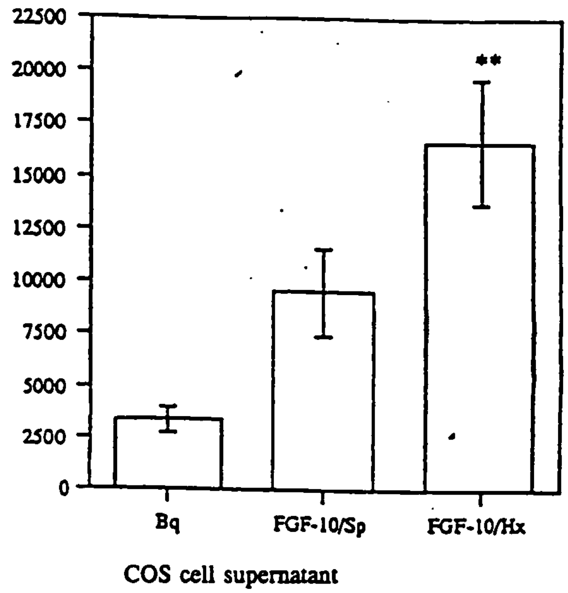
[Figure 5]



(A) micrograph of ultrathin slice specimen of joint cartilage tissue

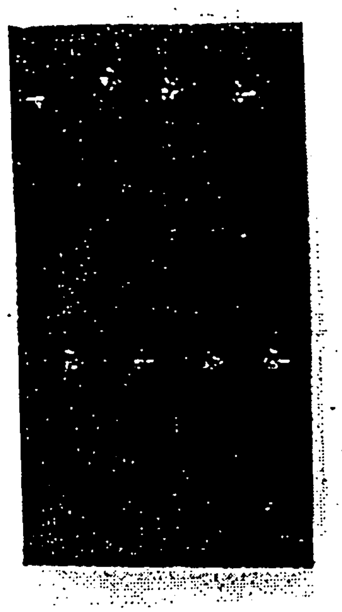
(B) micrograph of ultrathin slice specimen of apophysis cartilage tissue
Photos replacing figures

[Figure 6]



Changes of the incorporation of tritium-labeled thymidine in FRSK cells after the addition of COS cell supernatant
Values are mean ± standard deviations. $p < 0.01$ is considered significant (**), compared to Bq.

[Figure 7]
Processed image of soft X-ray picture of tibia in human FGF-10 administration group in Testing Example.
(Upper: 10.6 μg of FGF-10; lower: 21.2 μg of FGF-10)



[Figure 8]

Processed image of soft X-ray picture of tibia in control group in Testing Example.

Reference number 132317

[Document name] Summary
[Summary]
[Subject]

To provide a DNA encoding the fibroblast growth factor FGF-10 having a novel amino acid sequence, the recombinant protein, and a therapeutic agent for injuries of bone/cartilage.

[Composition]

Recombinant FGF-10 was obtained by transforming an expression vector containing the DNA encoding the specific amino acid sequence into a host cell and then culturing the resultant transformant to produce the protein. The therapeutic effect of the recombinant FGF-10 on injuries of bone/cartilage was confirmed.

[Effects]

FGF-10 can be used as a therapeutic agent and research reagent due to its cell-growth-promoting activity.

[Selected Figure] None

[Document name] Correction data

[Corrected document] Patent application

<Acknowledged information - additional information>

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